



NCI ETI Branch Flow Cytometry Core Laboratory

Simultaneous analysis of cell death, DNA content and surface immunophenotype using gentle ethanol fixation techniques.

A major issue in the simultaneous analysis of DNA content using DNA binding dyes and cell surface immunophenotyping using fluorochrome-conjugated antibodies is the choice of cell pretreatment technique. For successful DNA content measurement, cell and nuclear membranes need to be sufficiently permeablized to allow entry of the DNA binding dye. Ethanol or other alcohols have been traditionally used to permeablize cells for DNA content analysis; this treatment, however, has the disadvantage of damaging cell surface proteins, as well as causing dissociation of fluorochrome-conjugated antibodies from cell surface antigens. Crosslinking of cell surface proteins with paraformaldehyde, either by itself or prior to ethanol treatment, has been previously used in an attempt to circumvent this problem. Paraformaldehyde crosslinks chromatin, however, limiting stoichiometric binding of DNA probes and reducing the quality of the resulting DNA content distribution. In an attempt to accurately measure DNA content with simultaneous preservation of cell surface markers, we have utilized gentle ethanol treatment techniques, which permeablize cells with minimal loss of surface antigen expression and antibody-antigen association. For some cell types, the presence of apoptotic cells based on reduced DNA content can also be detected. One such technique employs the addition of ethanol to cells previously resuspended in high concentrations of fetal bovine serum, and has been thoroughly described in the literature.

Important note! This technique measures apoptosis by reduced DNA dye binding to chromatin, a phenomenon that can occur in situations other than apoptosis (such as necrotic cell death, mechanical cell damage, etc.). Assays of this type should therefore *never* be used by themselves as definitive indicators of cell death. Confirmation of apoptosis should always be carried using multiple assays for cell death, such as TUNEL, annexin V binding, caspase activation, etc. DNA dye binding assays may often be unsuitable for death determination in certain cell and tissue types, depending on the cell death process at work.

Materials

- The fluorochrome-conjugated antibody or antibodies of interest.
- An appropriate DNA binding dye. For single phenotype analysis, **propidium iodide** can be used simultaneously with FITC-conjugated antibodies for analysis on a single laser flow cytometer. Analysis of two phenotypes on a single laser instrument can be accomplished using FITC- and PE-conjugated antibodies and the far red DNA binding dyes **7-aminoactinomycin D** or **LDS-751**. **Hoechst 33342** and **33258** or **DAPI** can be used on dual laser (488 nm/UV) instruments for simultaneous analysis with two or three fluorochrome-conjugated antibodies. RNase should be added for DNA dyes that can bind dsRNA (such as propidium iodide).
- 70% EtOH in ddH₂O
- 50% fetal bovine serum (FBS) in PBS with 0.1% sodium azide

- 70% EtOH in glycerol
- staining buffer (2% FBS in PBS with 0.1% sodium azide)

Procedure

- Prepare the cell type of interest as a single cell suspension and wash once with cold PBS with 0.1% sodium azide. Label with the fluorochrome-conjugated antibody of interest in cold staining buffer.
- Wash cells once with staining buffer and once with PBS/azide alone. Decant the supernatant, shake tube gently to resuspend pellet and add 1 part (i.e. 0.3 mls) 50% FBS in PBS. While gently mixing, add 3 parts (i.e. 0.9 mls) cold 70% EtOH in ddH₂O dropwise. Some precipitation of the FBS proteins will occur during addition of ethanol; this can be disregarded, as these precipitates will be removed during subsequent washes. Incubate for at least two hours or overnight at 4°C.
- Wash the cells twice with cold PBS/azide to remove EtOH and precipitated protein and add propidium iodide at 50 m g/ml in PBS with 100 U/ml DNase-free RNase. Other DNA content dyes can be substituted for propidium iodide at this point. 7-aminoactinomycin D can be used at concentrations between 10 and 25 m g/ml, and Hoechst 33342 and 3258 and DAPI at 1 to 2 m g/ml, for example. Analyze using the appropriate instrument.
- Preliminary evidence suggests that 70% EtOH in glycerol can be substituted for 50% FBS followed by 70% EtOH addition with similar preservation of surface markers.

References

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